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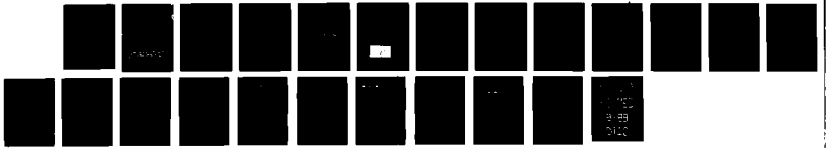

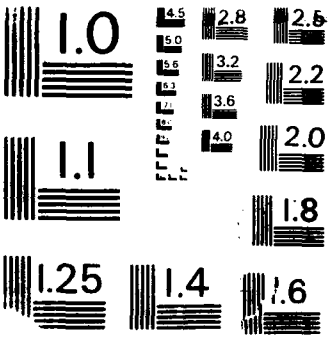
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The goals of our research were to investigate the molecular mechanisms controlling the expression of luminescence genes (*luxR*, *luxICDABE*) of the symbiotic bioluminescent bacterium *Vibrio fischeri* and to identify and investigate the regulation of other genes potentially involved in the symbiosis of this bacterium with monocentrid fish. The work has defined: 1) positive transcriptional control of *lux* expression by cAMP-CRP and negative transcriptional control of *lux* expression by autoinducer-LuxR; 2) a requirement for cAMP-CRP for induction of luminescence in regulatory mutants (*cya* like and *crp* like mutants) of *V. fischeri*; 3) an involvement of cAMP-CRP in iron regulation of luminescence in *V. fischeri*; and 4) a gene transfer system for use in analysis of *lux* gene expression and expression of other potentially symbiosis-related genes in *V. fischeri*.

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TABLE OF CONTENTS

Title: "*Vibrio fischeri* Symbiosis Gene Regulation"

PROJECT GOALS

The goals of this project were to investigate the molecular mechanisms controlling luminescence gene expression of the symbiotic bioluminescent bacterium *Vibrio fischeri*; and to identify and investigate the regulation of other symbiosis functions in this marine bacterium.

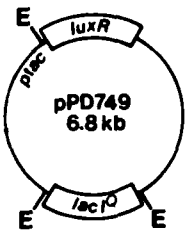
WORK COMPLETED

A. Transcriptional control of *luxR* expression by cAMP-CRP and LuxR

Evidence to date indicates that the *luxR* gene product is the transcriptional activator of the *V. fischeri luxICDABE* genes (genes for an autoinducer synthesis enzyme and luminescence enzymes). As such, a knowledge of the regulatory factors that control *luxR* expression is essential for understanding *lux* gene regulation both in general terms and in detail. Previous studies had indicated that cyclic AMP (cAMP) and cAMP receptor protein (CRP) are required for induction of the *luxICDABE* genes in *E. coli*, as monitored with luciferase (*luxAB* gene product), and that cAMP-CRP appeared to function in the *lux* regulon by activating transcription from the *luxR* promoter, as monitored with β-galactosidase from a Mu dI (*lacZ*) insertion in *luxR*. However, these studies were conducted in the absence of a functional *luxR* gene (due to insertional inactivation by Mu dI) and with a single *lacZ*::*luxR* fusion.

To address these caveats and to better understand the mechanism of transcriptional control of *luxR*, a *luxR* complementation plasmid (pPD749) was constructed by sub-cloning *luxR* under control of the cAMP-CRP-independent *tac* promoter to provide LuxR in trans under control of IPTG (Fig. 1A). Additional *lacZ*::*luxR* fusions were generated using Mu dI1734, and four that mapped at widely separated positions in *luxR* (Fig. 1B) were examined in the complementation system.

A.



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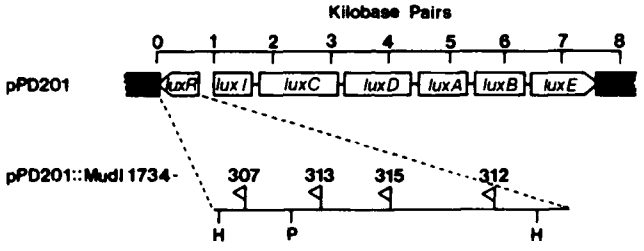

FIG. 1. Two plasmid *lux* complementation system. A) pPD749, containing *luxR* under control of the *tac* promoter and with the *lacI* gene for IPTG control of the *tac* promoter. B) Organization of the *lux* genes and map locations of the four *luxR*::Mu dI1734 (*lacZ*) fusions used to study *luxR* promoter activity. E, EcoRI; H, HindIII; P, PstI. The flags indicate the positions of the Mud I1734 insertions and point in the direction of *lacZ* transcription.

Studies with these recombinant *lux* plasmids in *E. coli* adenylate cyclase (*cya*) and CRP (*crp*) mutants revealed: 1) that in the presence of high levels of the *luxR* gene product (that is, LuxR expressed from pPD749), cAMP and CRP are no longer necessary for induction of luminescence and luciferase synthesis (i. e., transcription of *luxICDABE*); 2) that cAMP and CRP activate transcription from the *luxR* promoter regardless of the presence or absence of a functional *luxR* gene and regardless of the position of the *lacZ* fusion in *luxR*; and strikingly, 3) that the *luxR* gene product negatively autoregulates transcription from the *luxR* promoter. These results are interpreted as indicating that cAMP-CRP function indirectly in the *lux* regulon by activating transcription from the *luxR* promoter. This activation leads to an increase in the level of LuxR in the cells, thereby potentiating the system for autoinduction. These studies have led to a working model for *lux* gene regulation that includes this role for cAMP-CRP and the role of LuxR in *luxR* negative autoregulation (Fig. 2).

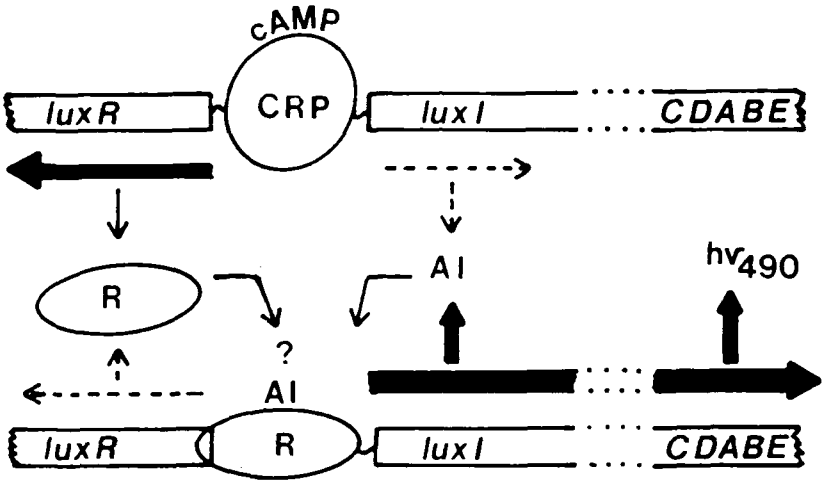

FIG. 2. Model for transcriptional regulation of the *V. fischeri lux* regulon. CRP and cAMP bind to the region between *luxR* and *luxI*, activate transcription of *luxR* (indicated by the heavy arrow) and decrease transcription of *luxICDABE* (indicated by the very light arrow). The activation of *luxR* transcription increases the concentration of the LuxR protein (R) to a sufficient level for interaction with the low concentration of autoinducer (AI) that has accumulated due to basal expression of the *luxI* gene and the resulting low activity of the *luxI* gene product. The LuxR protein and autoinducer then bind to a region upstream of the *luxICDABE* promoter, activating transcription of *luxICDABE*. This leads to increased levels of autoinducer and to induced levels of luminescence ($h\nu_{490}$).

B. Role of *Vibrio fischeri* autoinducer in *luxR* negative autoregulation

According to the current working model for *lux* gene regulation (Fig. 2), autoinducer plays an essential role in transcriptional activation of *luxICDABE*. It is not clear from the studies described above, however, whether autoinducer is actually involved in the observed transcriptional negative autoregulation of *luxR* expression, although the data suggest this. Cells in the above studies contained an intact *luxI* (autoinducer synthesis) gene, enabling them to produce autoinducer during the course of the experiments. Consequently, the presence and amount of autoinducer could not be controlled. To more rigorously address the question of the role of autoinducer in *luxR* negative autoregulation, we (P.V. Dunlap and J. M. Ray) have generated non-polar mutations in the cloned *luxI* gene so that experiments similar to those described above could be conducted in the absence of autoinducer and with controlled amounts of autoinducer. To accomplish this, we made point mutations in the *lux* genes, using an in vitro hydroxylamine mutagenesis procedure with the cloned *lux* system, and then screened the mutated plasmids in *E. coli* using a two-plasmid complementation

protocol to identify those mutations occurring in *luxI*. Our completed studies with one such *luxI* mutant have demonstrated conclusively that *luxR* negative autoregulation requires the presence of autoinducer and occurs concommitantly with autoinducer-LuxR activation of the divergently transcribed *lux* operon. The strain containing the *luxI* mutation is a very sensitive tool for autoinducer assays and has already found application in studies of the pathway of autoinducer synthesis by other researchers.

C. Control of luminescence in regulatory mutants of *Vibrio fischeri*

Much of the existing information on cAMP-CRP control of luminescence has been derived from studies with *E. coli* containing recombinant *lux* plasmids. While the results of these studies provide a consistent and interpretable view of *lux* gene regulation, the fact that these studies were carried out in a heterologous background with multi-copy plasmids could pose interpretational problems due to possible artefacts in the observed regulation. Thus, in the absence of comparable studies with mutants of *V. fischeri*, the validity of these studies of cAMP-CRP control of *lux* gene expression in *E. coli* remains subject to question. At the time the work with *E. coli* was initiated, there were no cAMP and CRP mutants of *V. fischeri* with which to addess this problem.

Consequently, to resolve this problem, I have used a phosphomycin selection procedure to isolate spontaneous mutants of *V. fischeri* that are apparently deficient in adenylate cyclase (*cya*-like) and CRP (*crp*-like). The isolation procedure required several modifications to accomodate the growth requirements of *V. fischeri* and its high sensitivity to components of indicator media. Despite these problems, a successful procedure was developed. The isolated mutants exhibited several characteristics comparable to *cya* and *crp* mutants of *E. coli* and *Salmonella typhimurium*, including a pleiotropic carbohydrate negative phenotype, a decreased sensitivity to antibiotics thought to be transported by cAMP-CRP-controlled systems, and altered levels of cellular cAMP. Notably, compared to the wild-type strain, the mutants produced a very low level of luminescence and luciferase in the absence of added cAMP. Addition of cAMP to the growth medium, with or without added autoinducer, restored luminescence and luciferase synthesis in the *cya*-like mutant to levels approaching those of the wild-type strain, but had no effect on luminescence or luciferase synthesis in the *crp*-like mutant (see Fig. 3). Addition of autoinducer alone effected a small increase in luminescence and luciferase synthesis in both mutants. These results demonstrate that cAMP and CRP are required for the autoinduction of luminescence in *V. fischeri*, and they are very similar to results obtained with *E. coli cya* and *crp* mutants containing the *V. fischeri lux* genes on recombinant plasmids. Thus, these results confirm earlier conclusions for cAMP-CRP control of luminescence.

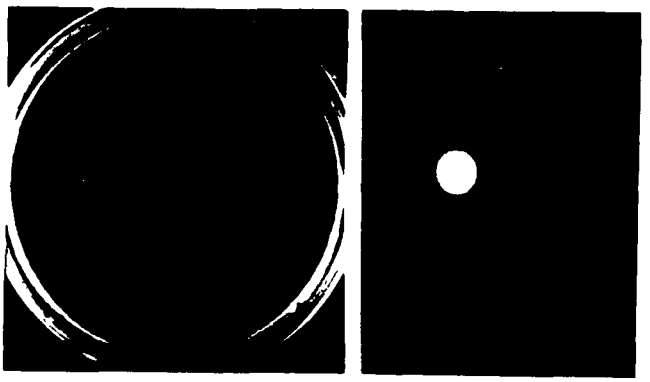

FIG 3. Luminescence response of *V. fischeri cya*-like and *crp*-like mutants to cAMP. (Left panel) *cya*-like mutant on the left and *crp*-like mutant on the right, photographed in the light approximately 4 hrs after addition of cAMP to cells. (Right panel) Same plate photographed in the dark.

Except for natural isolates of *V. fischeri* that are deficient in autoinducer synthesis, these *cya*-like and *crp*-like mutants are the first regulatory mutants of this species to be described. The isolation of these mutants should facilitate studies of *lux* gene regulation and other cAMP-CRP-controlled functions in *V. fischeri*.

WORK INITIATED

A. Mechanism of iron regulation of the *Vibrio fischeri* luminescence system

The use of the *V. fischeri cya*-like and *crp*-like mutants has unexpectedly revealed a requirement for cAMP and CRP in iron regulation of luminescence. In *V. fischeri*, it has been shown by other investigators that excess iron can repress luminescence, and limitation of iron availability stimulates luminescence and luciferase synthesis while restricting growth. The mechanism for iron regulation of luminescence is not known. To determine if cAMP and CRP are involved in this iron regulation, I have utilized the *cya*-like and *crp*-like mutants of *V. fischeri* described above. Addition of the iron chelator ethylenediamine di(o-hydroxyphenyl acetic acid) (EDDHA) was found to restrict growth rate in both mutants (as it does in the wild-type strain), but it stimulated luminescence only in the *cya*-like mutant and only in the presence of added cAMP. I interpret these results as indicating that the stimulated expression of the *lux* system by iron-limiting growth conditions requires cAMP and CRP (P.V. Dunlap, ASM abstract H177, 1988). To gain more insight into the mechanism of iron regulation of luminescence and the relationship between cAMP and iron, I have carried out experiments with *E. coli* iron transport mutants transformed with recombinant *lux* plasmids and with *V. fischeri* strains, grown under iron-limiting and iron-excess conditions. The details of this work will be provided in a publication that is in preparation.

B. Development of a gene transfer system for *Vibrio fischeri*

To effectively study control of the *lux* system in *V. fischeri* and to carry out studies on other possible symbiosis functions in this organism, a means to transfer DNA into *V. fischeri* is essential. No procedure for transforming, transducing or conjugating DNA into *V. fischeri* has yet been described. Consequently, we have concentrated on this problem, and we have made substantial progress in the development of a gene transfer system for *V. fischeri*. This system is being used to define aspects of luminescence gene regulation in *V. fischeri* and to create random transposon-generated mutations in the *V. fischeri* chromosome for identification of other sets of genes potentially involved in the symbiosis. Details of this work will be presented in a publication that is in preparation.

PERSONNEL

One research assistant (female caucasian, U.S. citizen) and two undergraduate students (one male caucasian, citizen of the Federal Republic of Germany, permanent U.S. resident; and one female caucasian, U.S. citizen) were supported on this contract.

PUBLICATIONS AND RESEARCH PRESENTATIONS

Published Articles: (Reprints attached)

Dunlap, P.V. and E.P. Greenberg. 1988. Control of *Vibrio fischeri lux* gene transcription by a cAMP receptor protein-LuxR protein regulatory circuit. J. Bacteriol. **170**:4040-4046.

Dunlap, P. V. 1989. Regulation of luminescence by cyclic AMP in cya-like and crp-like mutants of *Vibrio fischeri.* J. Bacteriol. **171**:1199-1202.

Dunlap, P. V., and J. M. Ray. 1989. Requirement for autoinducer in transcriptional negative autoregulation of the *Vibrio fischeri luxR* gene in *Escherichia coli.* J. Bacteriol. **171**:3549-3552.

Manuscripts in Preparation: (The following papers are in preparation and result in part from studies initiated during the contract.)

Dunlap, P. V. 1989. Iron regulation of *Vibrio fischeri* luminescence gene expression. (In preparation).

Dunlap, P. V. 1989. Development of a gene transfer system for analysis of luminescence gene regulation in the marine symbiotic bacterium *Vibrio fischeri.* (In preparation).

Abstracts and Research Presentations:

Dunlap, P. V. 1987. Regulation of *Vibrio fischeri lux* gene expression in *Escherichia coli*: role of cAMP-CRP in *luxR* transcriptional activation and negative autoregulation. New Mexico Branch, Amer. Soc. Microbiol. Ann. Meet., Albuguerque, NM (October 30-31).

Dunlap, P. V. 1988. Regulation of luminescence by cyclic AMP and iron in Cya and Crp mutants of *Vibrio fischeri.* New Mexico Branch, Amer. Soc. Microbiol. Ann. Meet., Las Cruces, NM (October 14-15).

Dunlap, P. V., and J. M. Ray. 1988. Role of autoinducer in negative autoregulation of *luxR*, the gene encoding the transcriptional activator of the *Vibrio fischeri* luminescence system. New Mexico Branch, Amer. Soc. Microbiol. Ann. Meet., Las Cruces, NM (October 14-15).

Dunlap, P. V. 1988. Control of luminescence in *cya*-like and *crp*-like mutants of*Vibrio fischeri.* Abstract #H177, 88th Ann. Meet. Amer. Soc. Microbiol. (Miami Beach, FL, May 8-13), pg. 174.

Dunlap, P. V. 1989. Iron regulation of *Vibrio fischeri lux* gene expression in *Escherichia coli.* Abstract #H34, 89th Ann. Meet. Amer. Soc. Microbiol., (New Orleans, LA, May 14-18), pg. 175.

Invited Research Presentations:

Department of Biology, Woods Hole Oceanographic Institution, Woods Hole, MA (June 16, 1988): Cyclic AMP Control of Luminescence in the Symbiotic Bacterium *Vibrio fischeri.*

Department of Biological Sciences, University of Nevada, Las Vegas, NV (September 30, 1988): Regulation of Bioluminescence in the Marine Symbiotic Bacterium *Vibrio fischeri.*

Instituto de Investigaciones Bioquimicas, Fundacion Campomar, Buenos Aires, Argentina (January 6, 1989): Regulation of Luminescence Gene Expression in *Vibrio fischeri.*

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Regulation of Luminescence by Cyclic AMP in *cya*-Like and *crp*-Like Mutants of *Vibrio fischeri*

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Mutants of *Vibrio fischeri* MJ-1 (wild type) apparently deficient in adenylate cyclase (*cya*-like) or cyclic AMP receptor protein (*crp*-like) were isolated and characterized. Compared with MJ-1, the mutants produced low levels of luminescence and luciferase. Addition of cyclic AMP restored wild-type levels of luminescence and luciferase in the *cya*-like mutant but not in the *crp*-like mutant. The results are consistent with the hypothesis that in *V. fischeri* cyclic AMP and cyclic AMP receptor protein are required for induction of the luminescence system.

Luminescence in *Vibrio fischeri*, a cardinal feature of its light organ symbiosis with monocentrid fish, requires autoinduction. During growth in culture, *V. fischeri* produces a specific, diffusible metabolite called autoinducer [*N*-(3-oxohexanoyl)homoserine lactone] which accumulates in the medium and induces synthesis of luciferase and other enzymes involved in luminescence when it reaches the critical concentration of a few molecules per cell (11, 20, 25, 30). In natural environments in which the *V. fischeri* population density can reach a high level, such as the fish light organ (10^9 to 10^{10} cells per ml of organ fluid), autoinducer presumably also accumulates and induces luminescence (29, 30). Recently, a 9-kilobase fragment of *V. fischeri* DNA containing genes specifying the luminescence enzymes and encoding regulatory functions necessary for their expression in *Escherichia coli* was isolated (12). The luminescence (*lux*) system contains seven genes in two divergently transcribed units (*luxR* and *luxICDABE*) (5, 12, 13). Expression of the *lux* genes in *E. coli* is autoinducible in a fashion analogous to that in *V. fischeri* (9, 12), with expression dependent on an apparently complex autoregulatory circuitry (9, 10, 12–14, 21).

Besides control by autoinduction, luminescence in *V. fischeri* is subject to an apparently atypical catabolite repression. Glucose represses luminescence transiently in batch culture; however, this repression is not reversed by cyclic AMP (cAMP), and prior growth of *V. fischeri* on glucose eliminates the repression (30). In contrast, in phosphate-limited chemostat culture, cAMP reversal of glucose repression has been demonstrated (16). Additionally, since the luminescence system is not involved in transport or catabolism of a carbon substrate, it is not obvious why cAMP should be involved in its control. Moreover, it has been postulated that glucose is the carbon source supplied to *V. fischeri* by its fish host (27).

Recently, studies with the cloned *V. fischeri lux* genes in *E. coli* adenylate cyclase (*cya*) and cyclic AMP receptor protein (CRP) (*crp*) mutants have addressed this controversy by demonstrating that autoinduction of luminescence requires cAMP and CRP, even though glucose repression of luminescence is not reversed by exogenous cAMP in the parental (*cya*$^+$ *crp*$^+$) strain (9, 10). The presence of a possible CRP-binding site within the *lux* regulatory region, identified by DNA sequence analysis (7, 15), supports these studies. However, evidence for cAMP-CRP control of luminescence obtained with *V. fischeri* regulatory mutants has not been reported.

Isolation and characterization of *V. fischeri cya*-like and *crp*-like mutants. To address questions of the role of cAMP-CRP in luminescence in *V. fischeri*, spontaneous mutants of a wild-type strain of *V. fischeri* (MJ-1) (30), apparently deficient in adenylate cyclase (*cya*-like) or CRP (*crp*-like), were isolated by a modification of the phosphomycin selection procedure (1, 23). *V. fischeri* MJ-1 was grown overnight with aeration at 22°C in the minimal medium of Nealson (26) supplemented with glucose (10 mM, final concentration), glucose 6-phosphate (20 mM), and DL-α-glycerophosphate (0.6 mM). Cells (0.1 ml; approximately 5×10^8 cells) were then plated on a selective medium, based on Luria-Bertani (LB) medium (31), which contained (per liter of tap water) 10 g of tryptone, 5 g of yeast extract, 0.34 M NaCl, 20 mM glycerol, 20 mM Tris hydrochloride (pH 7.5), and 15 g of agar (LB-salt medium [LBS]). The LBS was modified to include neutral red (30 μg · ml^{-1}) (LBS-NR), galactose and maltose (25 mM each), and 1.5 mM phosphomycin. After 3 to 5 days of incubation, colonies that exhibited a phenotype consistent with a defect in adenylate cyclase or in CRP (i.e., slow growth, no fermentation of the sugars [color of colonies, gold to pale pink, compared with magenta for the wild-type strain], and little or no visible luminescence) were picked and purified on fresh selection plates. In a preliminary screening of 20 of these strains for their response to cAMP, 15 strains (*cya*-like) fermented galactose and maltose and produced high levels of luminescence on LBS-NR plates (without phosphomycin) when cAMP was added; 5 strains (*crp*-like) did not.

Representatives of each type of mutant (MJ-16 [*cya*-like] and MJ-46 [*crp*-like]) were examined in detail. Fermentation of carbon sources was tested on LBS-NR plates, with carbon sources filter sterilized and added to a final concentration of 25 mM to the autoclaved, cooled medium. Like MJ-1 (wild type), MJ-16 and MJ-46 fermented D-glucose, D-fructose, and *N*-acetylglucosamine in the absence of added cAMP; unlike the wild-type strain, however, the mutants did not ferment D-galactose, maltose, D-mannose, D-cellobiose, or D-glucose 6-phosphate. Addition of cAMP (10 μl, 0.5 M stock, spotted over cells grown on the LBS-NR plates for 12 h) restored the ability of MJ-16 to ferment this latter group of sugars but had no effect on their fermentation by MJ-46. Consistent with these results, MJ-16 and MJ-46

TABLE 1. Growth inhibition of *V. fischeri cya*-like and *crp*-like mutants by antibiotics

Antibiotic and concn	Inhibition zone (mm)[a]				
	MJ-1 (wild type)	MJ-16 (*cya*-like)		MJ-46 (*crp*-like)	
		−cAMP	+cAMP[b]	−cAMP	+cAMP[b]
Phosphomycin					
25 μg	2.0	0	1.0	0	0
250 μg	9.0	2.0	4.5	1.5	1.5
2.5 mg	13.0	5.0	10.0	5.0	5.0
Streptomycin					
25 μg	0.1	0	0.1	0	0
250 μg	4.0	2.0	3.0	3.2	3.2
2.5 mg	9.5	7.0	9.0	8.0	8.0

[a] Sterile filter paper disks were saturated with 25 μl of each antibiotic (at concentrations of 1, 10, and 100 mg/ml) and placed over lawns of cells spread on LBS plates. Inhibition zones, measured as the distance from the edge of the disk to the edge of visible growth, were determined after incubation of plates at 22°C for 72 h.

[b] Final concentration of 10 mM.

grew on minimal plates containing D-glucose, D-fructose, or *N*-acetylglucosamine, as did MJ-1, but unlike MJ-1, MJ-16 and MJ-46 did not grow on minimal plates containing D-galactose, maltose, D-mannose, D-cellobiose, D-glucose 6-phosphate, D-ribose, D-mannitol, glycerol, or DL-α-glycerophosphate. Thus, the *V. fischeri* mutants exhibited a pleiotropic carbohydrate-negative phenotype that in one strain (MJ-16) but not in the other (MJ-46) could be reversed by the addition of cAMP to the growth medium. The altered fermentation patterns of MJ-16 and MJ-46 make it unlikely that a mutation other than one in the putative *cya* or *crp* loci was affecting expression of the luminescence system (see below).

The antibiotics phosphomycin and streptomycin are thought to be transported by cAMP-CRP-controlled systems and have been used for the isolation and characterization of *cya* and *crp* mutants of *E. coli* and *Salmonella typhimurium* (1, 2, 23). In the absence of added cAMP, both antibiotics inhibited the growth of the mutants less than that of the wild-type strain (Table 1). The presence of cAMP, however, substantially increased the inhibition of MJ-16 but did not increase the inhibition of MJ-46 (Table 1).

Estimates of cAMP levels in the mutants and in the wild-type strain were determined by the method of Gilman (17), as modified by Botsford (3), with a binding-protein preparation from beef kidney (6). To extract cAMP, *V. fischeri* cells were grown in LBS-glucose broth to an optical density at 660 nm of 0.5 (approximately 3×10^8 cells · ml^{-1}, 100 μg of protein · ml^{-1}); pelleted by microcentrifugation (room temperature; 11,000 × *g* 1 min), rapidly suspended without washing in 0.4 ml of hot (95°C) sodium acetate buffer (50 mM, pH 4.0), and heated at 95°C for 10 min. The cell debris was then pelleted, and the extract was transferred to a clean tube and frozen at −20°C until it was assayed. cAMP was detected in MJ-1 (wild type) and MJ-46 (*crp*-like) at levels of 2.7 and 0.5 pmol per 10^7 cells, respectively, but was not detected in MJ-16 (*cya*-like). For comparison, *E. coli* PD100 (*cya*$^+$ *crp*$^+$), PD200 (Δ*cyaA*), and PD300 (Δ*crp*) (9) prepared similarly were found to contain 1.7, 0, and 9.3 pmol of cAMP per 10^7 cells, respectively. Thus, *V. fischeri* appears to contain cAMP, and at least for the wild-type strain and the *cya*-like mutant, the pattern of the presence and absence of cAMP is consistent with that found in *E. coli*. The detected levels of cAMP are higher than those typically reported for *E. coli* (e.g., reference 3), probably because substantial amounts of extracellular cAMP were associated with the unwashed cell pellets.

The mutants reverted spontaneously to the wild-type phenotype (i.e., utilization of sugars and production of luminescence) at frequencies of approximately 10^{-7} (MJ-16) and 10^{-8} (MJ-46), which are consistent with the defects being single point mutations.

Control of cellular luminescence and luciferase synthesis by cAMP. Compared with MJ-1 (wild type), the mutants produced a very low level of luminescence. To examine the luminescence response of the mutants to added nucleotides, cultures were grown for 12 to 18 h on LBS plates, and 10 μl of the appropriate nucleotide solution (0.5 M stock) was then spotted over the cells. Responses were noted periodically from 2 to 6 h and again 12 h after addition of the nucleotide by visual observation of the plates in a well-darkened room (10-min dark adaptation). Addition of cAMP restored a high level of luminescence in the *cya*-like mutant but had no effect in the *crp*-like mutant. The response to cAMP was specific: addition of adenine, adenosine, AMP, ADP, ATP, cGMP, dibutyryl cGMP, or dibutyryl cAMP did not stimulate luminescence in either MJ-16 or MJ-46.

To quantify the cAMP response and to examine the luminescence behavior of the mutants, cultures were grown in LBS broth containing D-glucose (10 mM) and supplemented with cAMP (10 mM) and autoinducer [*N*-(3-oxohexanoyl)homoserine lactone, 0.2 μM], in 3-ml volumes, as described previously (9). The light-measuring equipment and standard to calibrate the equipment have been described previously (9, 18), as have the procedures for measuring luminescence of broth cultures (9).

In the absence of added cAMP, the *cya*-like mutant (MJ-16) produced a low level of luminescence that increased with cell density during growth (Fig. 1). With added cAMP, however, with or without added autoinducer, luminescence reached levels similar to induced levels produced by the wild-type strain (Fig. 1). When autoinducer was added with cAMP, this fully induced level of luminescence was reached sooner (Fig. 1), presumably because the added autoinducer eliminated the delay that occurs while autoinducer produced by the cells accumulates to the critical concentration necessary for induction (5 to 10 nM) (20). Addition of autoinducer alone provided some stimulation of luminescence, but the maximum level of luminescence was about 100-fold less than the fully induced level reached in the presence of cAMP (Fig. 1).

For the *crp*-like mutant (MJ-46), addition of cAMP did not stimulate luminescence. Regardless of the presence of added cAMP, luminescence in this strain was low and was similar to that of the *cya*-like mutant grown in the absence of added cAMP, both with and without added autoinducer (Fig. 2).

Extracts of cells of MJ-16 and MJ-46 taken from the growth experiments described above were used to determine luciferase activity, as described previously (9, 10), with cells harvested from the cultures at an optical density at 660 nm of 1.4. Cell extracts were prepared as described previously (9), except that complete lysis of cells was obtained with one freeze-thaw cycle. The results were consistent with those for luminescence. Autoinducer alone effected a small increase in luciferase activity in both mutants, but luciferase levels approaching those of the wild-type strain were produced only by the *cya*-like mutant when grown in the presence of added cAMP (Table 2). Thus, cAMP and CRP appear to be

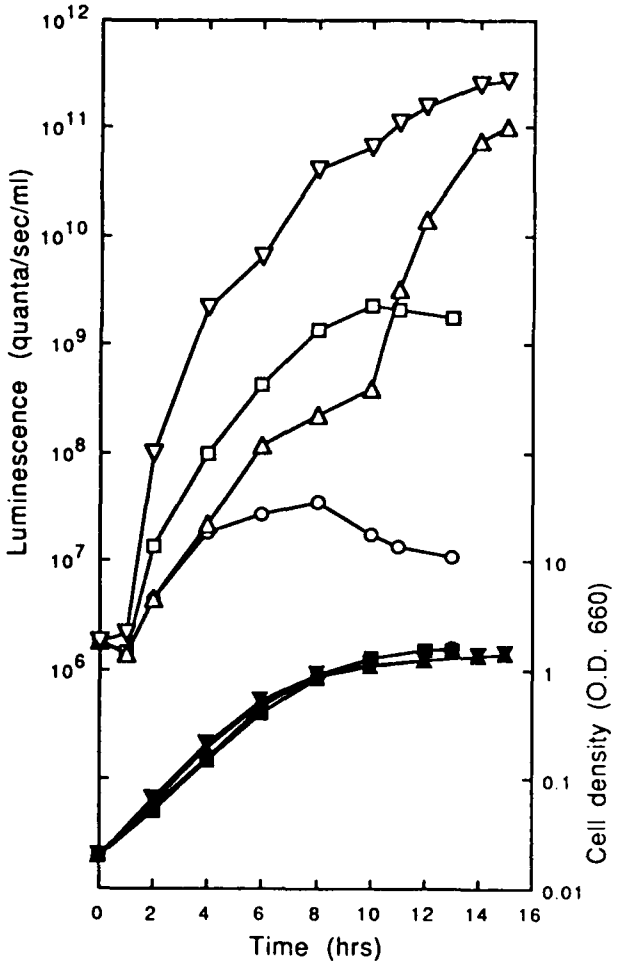

FIG. 1. Effects of cAMP and autoinducer on in vivo luminescence during growth of *V. fischeri* MJ-16 (*cya*-like). Open symbols, luminescence; solid symbols, cell density. Symbols: △, and ▲, cAMP added to the medium; □ and ■, autoinducer added; ▽ and ▼, autoinducer and cAMP added; ○ and ●, no addition. O.D. 660, Optical density at 660 nm.

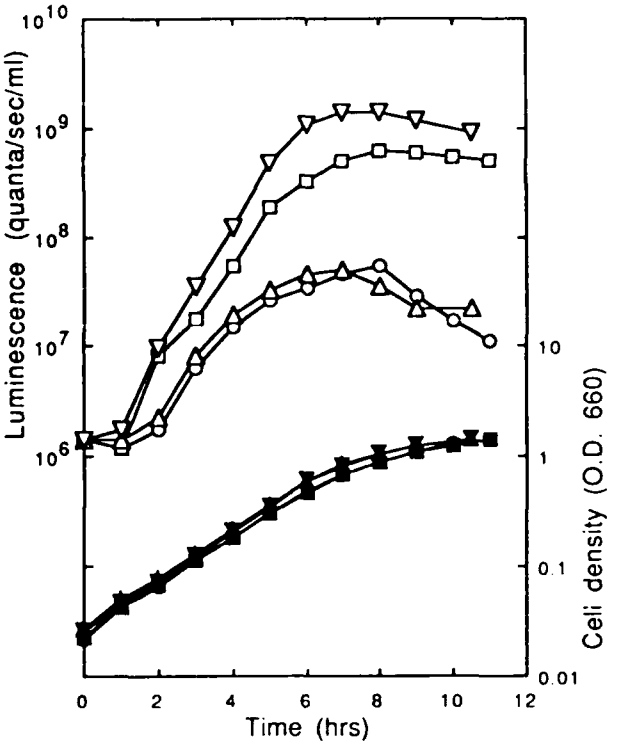

FIG. 2. Effects of cAMP and autoinducer on in vivo luminescence during growth of *V. fischeri* MJ-46 (*crp*-like). Symbols are as in the legend to Figure 1. O.D. 660, Optical density at 660 nm.

required for induction of luminescence and luciferase synthesis in *V. fischeri*.

These results confirm studies with *E. coli cya* and *crp* mutants containing recombinant *lux* plasmids in which cAMP and CRP were shown to be required for induction of the *lux* system (9, 10) and studies with the wild-type *V. fischeri* in which cAMP control of luminescence was implicated (16). With regard to the altered patterns of carbohydrate fermentation and antibiotic sensitivity, the *V. fischeri* mutants are similar to *E. coli* and *S. typhimurium cya* and *crp* mutants. Phenotypically, MJ-16 appears to be deficient in adenylate cyclase, and MJ-46 appears to be deficient in CRP. Furthermore, the luminescence and luciferase responses of the mutants to cAMP and autoinducer (Fig. 1 and 2 and Table 2) are remarkably similar to the responses of *E. coli cya* and *crp* mutants containing the cloned *lux* genes on recombinant plasmids (9, 10). However, despite these similarities, in the absence of information on adenylate cyclase and CRP, the identification of these mutants as *cya*-like and *crp*-like should be considered tentative. This is particularly the case for MJ-46, since its *crp*-like identification is based on negative results and since, unlike *E. coli crp* mutants (4), it does not appear to overproduce cAMP. A pattern of responses like that of MJ-46 could derive from a mutation producing a very active 3′,5′-cAMP phosphodiesterase. At present, methods for genetic analysis of *V. fischeri* are not sufficiently developed to permit a genotypic characterization of these mutants.

Catabolite repression of luminescence in a closely related species, *Vibrio harveyi*, has been described elsewhere (28), and a mutant of this species that requires cAMP for induction of luminescence and for utilization of a variety of carbohydrates has been isolated (32). DNA sequence analysis of the cloned *V. harveyi lux* genes has revealed a consensus CRP-binding site in the putative *lux* regulatory region (24). Thus, similarities may exist in cAMP-CRP control of the *lux* systems of *V. fischeri* and *V. harveyi*. For other species of luminous bacteria, less information is available, but control of *lux* gene expression may involve cAMP and CRP (8, 19, 22).

Previous studies on the control of luminescence in *V. fischeri* have been hampered by the lack of regulatory

TABLE 2. Luciferase activity in *V. fischeri cya*-like and *crp*-like mutants

Addition(s) (concn)	Luciferase activity (10^5 U)[a]		
	MJ-1 (wild type)	MJ-16 (*cya*-like)	MJ-46 (*crp*-like)
None	2,300	45	40
Autoinducer (0.2 μM)	ND[b]	135	65
cAMP (10 mM)	ND	1,000	20
Autoinducer and cAMP	ND	1,800	60

[a] Normalized to an optical density at 660 nm of 1.0.
[b] ND, Not determined.

mutants. Except for natural isolates of *V. fischeri* deficient in autoinducer synthesis (25), the *cya*-like and *crp*-like mutants characterized here are the first regulatory mutants of this species to be described. Further studies of these mutants may lead to an understanding of the apparently atypical catabolite repression of luminescence in this species and should facilitate studies of *lux* gene regulation and other cAMP-CRP-controlled functions in *V. fischeri*.

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Requirement for Autoinducer in Transcriptional Negative Autoregulation of the *Vibrio fischeri luxR* Gene in *Escherichia coli*

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The effect of a mutation in *luxI* (autoinducer synthetase gene) on transcription of *luxR* in the cloned *Vibrio fischeri lux* system (*luxR*, *luxICDABE*) was examined in *Escherichia coli*. For the *luxI* mutant, transcription from the *luxR* promoter (monitored with β-galactosidase levels from a *luxR::lacZ* fusion, with LuxR supplied in *trans*) decreased fivefold, to levels of the *luxI*$^+$ strain, only in the presence of added autoinducer. The results demonstrate that, as has been shown at the translational level, autoinducer is required for negative autoregulation of *luxR* at the transcriptional level.

The expression of luminescence in *Vibrio fischeri*, a central aspect of its light organ symbiosis with monocentrid fish, is controlled by autoinduction. During growth in culture, *V. fischeri* produces a specific, diffusible metabolite termed autoinducer [*N*-(3-oxo-hexanoyl)homoserine lactone], which accumulates in the medium and induces synthesis of luciferase and other enzymes involved in luminescence when it reaches a critical concentration of a few molecules per cell (6, 15, 18, 20). In natural environments where the *V. fischeri* population density can reach a high level, such as in the fish light organ (10^9 to 10^{10} cells · ml of organ fluid $^{-1}$), autoinducer presumably also accumulates and induces luminescence (19, 20).

A 9-kilobase fragment of *V. fischeri* DNA that encodes all the functions for luminescence and contains regulatory elements sufficient for their expression in *Escherichia coli* has been isolated (8). The luminescence genes (*lux* genes) comprise two transcriptional units, *luxR* and the *lux* operon (*luxICDABE*), which are divergently transcribed from an intermediate regulatory region (8). In the *lux* operon, *luxI* encodes an autoinducer synthetase function and is the only *V. fischeri* gene necessary for synthesis of autoinducer in *E. coli*; *luxC*, *luxD*, and *luxE* specify proteins involved in synthesis of the aldehyde substrate for luciferase; and *luxA* and *luxB* encode the α and β subunits of luciferase (1, 8, 9). The *luxR* gene encodes a protein (LuxR protein) which is necessary for cells to respond to autoinducer (8). LuxR protein is thought to form a complex with autoinducer that activates transcription of the *lux* operon, thereby creating a positive feedback circuit for autoinducer synthesis which leads to an exponential increase in luminescence (8).

Recent evidence indicates that the expression of *luxR* is controlled both positively and negatively. With regard to positive control, cyclic AMP (cAMP) and cAMP receptor protein (CRP) activate transcription from the *luxR* promoter (4, 5), and this apparently accounts for the requirement for cAMP and CRP in induction of luminescence in *V. fischeri* (3, 12) and *E. coli* (4). A characteristic cAMP-CRP-binding site in the *lux* regulatory region has been identified by DNA sequence analysis (2, 11). Expression of *luxR* is controlled negatively at the level of translation by the *luxR* gene product, and this translational negative autoregulation is dependent on autoinducer (10). Expression of *luxR* also is subject to a negative control by the *luxI* gene product, and recent evidence indicates that this form of control involves a regulatory region in the *luxR* structural gene (9, 16).

In addition to the translational form of negative autoregulation, the expression of *luxR* is subject to negative autoregulation at the transcriptional level (5). During activation of *lux* operon transcription, the *luxR* gene product appears to counter the effect of cAMP-CRP by repressing transcription from the *luxR* promoter (5). However, whereas autoinducer is required for translational negative autoregulation of *luxR* (10), its involvement in transcriptional negative autoregulation has not been conclusively demonstrated (5). This report describes the use of a *luxI* mutation in the cloned *lux* genes to demonstrate that autoinducer is required for negative autoregulation of *luxR* at the transcriptional level.

Isolation and characterization of a *luxI* mutation. To generate mutations in the cloned *lux* genes, the in vivo hydroxylamine mutagenesis procedure of Silhavy et al. (21) was used, as modified by Engebrecht and Silverman (9). For this study, the recombinant *lux* plasmid pHK555 (*luxR::lacZ luxICDABE*) (5, 16) was used to permit assessment of *luxR* promoter activity through measurement of β-galactosidase levels in *E. coli* PD100 (*cya*$^+$ *crp*$^+$) and PD200 (Δ*cyaA*) (4). The *luxR* gene product was provided in *trans* from the *ptac* vector pPD749 under control of isopropyl-β-D-galactopyranoside (IPTG) (5). The solid medium used was Luria Bertani (LB) (21) agar (15 g · liter $^{-1}$). Growth of liquid cultures was in 3-ml volumes of LB broth containing Tris buffer (50 mM, pH 7.5) and glucose (10 mM) and, as indicated, ampicillin (80 μg · ml $^{-1}$), chloramphenicol (30 μg · ml $^{-1}$), and IPTG (1 mM), as described previously (4). All cultures were grown at 30°C. The light-measuring equipment, the standard to calibrate the equipment, and procedures for measuring luminescence in broth cultures and luciferase activity in cell extracts have been described (4, 14).

For mutagenesis, 15 μg of purified pHK555 was incubated in 50 μl of mutagenesis solution (9). Samples (5 μl) were removed periodically for 8 h, added to 95 μl of 100 mM $CaCl_2$ to stop the reaction, and stored at 4°C. This DNA was then used to transform *E. coli* PD100(pPD749) by the Hanahan procedure (13). Transformants were selected on LB plates containing ampicillin and chloramphenicol. To screen for strains with mutations in the *lux* genes, transformants were patched onto LB plates containing ampicillin, chloramphenicol, and IPTG. Those that produced little or no visible

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light (less than 20% of the light produced by cells containing pPD749 and unmutagenized pHK555) were classified as *lux* mutants. By this procedure, approximately 6% of the transformants were *lux* mutants.

Strains with mutations in aldehyde synthesis functions (*luxC*, *luxD*, and *luxE*) (1, 9) were eliminated by screening *lux* mutants for stimulation of luminescence by exposure to tetradecanal (5 μl spotted on inner surface of petri plate lid). Strains with mutations in *luxA* or *luxB* or with polar mutations in *luxI* or farther downstream in the *lux* operon were eliminated by screening for strains that produced little or no luciferase even when grown in the presence of pure, synthetic *V. fischeri* autoinducer (0.2 μM).

The remaining strains, which produced high levels of luminescence only in the presence of added autoinducer, were considered nonpolar *luxI* mutants. From one such strain, the mutagenized derivative of pHK555 (designated pJR551) was segregated away from pPD749 by transformation of PD100 and selection on LB plates containing only chloramphenicol. No changes in fragment sizes compared with those of pHK555 were detected with *Sal*I restriction endonuclease analysis, indicating no substantial physical changes in pJR551. PD100(pPD749) retransformed with segregated pJR551 also required autoinducer to produce high levels of luminescence. On LB plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (21), β-galactosidase levels of PD100(pPD749, pJR551) and PD100 (pPD749, pHK555) were essentially identical, indicating that mutagenesis of the plasmid did not generate an obvious defect in the *luxR* promoter or the fused *lacZ* gene.

To determine the sensitivity of PD100(pPD749, pJR551) to added autoinducer, we grew this strain in liquid culture supplemented with various concentrations of *V. fischeri* autoinducer. At low cell density (approximately 10^7 cells · ml^{-1}), the cells responded strongly to increasing concentrations of autoinducer (Fig. 1). Distinct responses were obtained at concentrations of 5 and 10 nM, which correspond to approximately 1 to 2 molecules of autoinducer per cell (15). The response of this strain was more sensitive and more consistent than that which we obtained with *V. fischeri* B-61, a natural isolate deficient in autoinducer synthesis (7, 15, 18). In addition, in purification and activity studies of autoinducer synthetase (*luxI* gene product), detectable responses have been obtained with PD100 containing pJR551 and pPD749 at an autoinducer concentration of 2 nM (A. Eberhard, personal communication). Thus, pJR551 is a sensitive tool for bioassays of *V. fischeri* autoinducer.

The sensitive luminescence response of this strain to exogenous autoinducer permitted a rigorous assessment of its ability to produce autoinducer. This ability was examined by determining the luminescence response of PD100 (pPD749, pJR551) cells inoculated into medium containing ethyl acetate extracts of medium conditioned by the growth of *E. coli* and *V. fischeri*. This method takes advantage of the fact that *V. fischeri* and *E. coli* containing the *V. fischeri lux* genes on recombinant plasmids release autoinducer into the medium during growth (6, 15, 18). Appropriate *E. coli* and *V. fischeri* strains (see below) were grown to a cell density at or beyond peak luminescence (optical density at 660 nm of 1.4 or above), the cells were removed, and the medium was extracted with ethyl acetate as described by Eberhard et al. (6). For response measurements, extracts were added to fresh medium to give a concentration equal to that of the conditioned medium. Luminescence (quanta · second^{-1} · milliliter^{-1} [10^8]), determined after 5 h of incubation, was as follows: control (unconditioned medium), 0.02; *E. coli* PD100(pPD749), 0.03; *E. coli* PD100(pPD749, pJR551) (*luxI* mutant), 0.03; *E. coli* PD100(pPD749, pHK555) (*luxI*$^+$), 225; *V. fischeri* B-61, 0.20. Thus, cells of PD100(pPD749, pJR551) responded strongly to the extract from PD100(pPD749, pHK555) with a 10,000-fold increase in luminescence compared with that of the control, and they responded slightly (a 10-fold increase) to the extract from *V. fischeri* B-61. However, the extract of self-conditioned medium [i.e., conditioned by PD100(pPD749, pJR551)] produced no response. Furthermore, an extract of self-conditioned medium concentrated 10-fold also produced no stimulation of luminescence. These results indicate that whereas PD100(pPD749, pJR551) responds strongly to either pure autoinducer or autoinducer extracted from conditioned medium, this strain does not produce autoinducer detectable by this sensitive bioassay. These results confirm the LuxI$^-$ phenotype of pJR551.

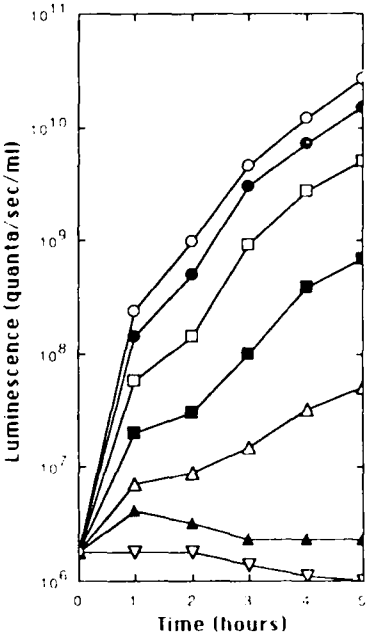

FIG. 1. Luminescence response of *E. coli* PD100(pPD749, pJR551) to *V. fischeri* autoinducer. Cells were inoculated at an initial density (optical density at 660 nm) of approximately 0.01. Growth of the cultures (not shown) was similar under all conditions. Symbols: ○, 200 nM; ●, 50 nM; □, 20 nM; ■, 10 nM; △, 5 nM; ▲, 2 nM; ▽, 0.2 nM or no autoinducer added.

Requirement for autoinducer in *luxR* negative autoregulation at the transcriptional level. In an earlier study, Engebrecht and Silverman (10) demonstrated a requirement for autoinducer in *luxR* negative autoregulation at the translational level. Recently, it was suggested that autoinducer also plays a role in *luxR* negative autoregulation at the transcriptional level (5). However, because the strains used in the latter study were able to produce autoinducer, its involvement at the transcriptional level could not be conclusively demonstrated (5). With the establishment of the LuxI$^-$ phenotype of pJR551, we were now in a position to assess the role of autoinducer in transcriptional negative autoregulation of *luxR*. For these studies, we transformed *E. coli* PD200 (Δ*cyaA*) (4, 5) containing pPD749 with pJR551 and pHK555 separately and compared the activities of the *luxR* and *lux* operon promoters of these two plasmids.

Consistent with previous results (4, 5), the presence of cAMP in the growth medium stimulated transcription from

TABLE 1. β-Galactosidase activity in *E. coli* PD200(pPD749) containing pJR551 or pHK555

Growth medium addition(s)	β-Galactosidase activity units	
	pJR551 (*luxI* mutant)	pHK555 (*luxI*$^+$)
None	65	45
cAMP (5 mM)	490	460
cAMP and autoinducer (0.2 μM)	350	360
cAMP and IPTG (1 mM)	450	170
cAMP, autoinducer, and IPTG	100	75

the *luxR* promoter in both strains (Table 1), as monitored with β-galactosidase levels (determined at an optical density at 660 nm of 1.0 by the $CHCl_3$-sodium dodecyl sulfate method of Miller [17]). However, in the *luxI* mutant [PD200(pPD749, pJR551)] grown in the presence of cAMP and IPTG (to induce synthesis of the LuxR protein from the p*tac* vector, pPD749), β-galactosidase levels decreased only slightly compared with the two- to threefold decrease in the *luxI*$^+$ strain [PD200(pPD749, pHK555)] (Table 1). However, when autoinducer was also added, the level decreased approximately fivefold in both the *luxI* mutant and the *luxI*$^+$ strain (Table 1). Similar results were obtained with the *cya*$^+$ strain, PD100 containing pPD749 and pJR551, without added cAMP. Thus, the presence of autoinducer is necessary for the LuxR protein-dependent decrease in expression from the native *luxR* promoter.

Activity of the *lux* operon promoter was monitored by assaying for luciferase in extracts of cells from the same experiment (Table 2). Consistent with results for PD100 described above (Fig. 1), induced levels of luciferase, reflecting induction of transcription from the *lux* operon promoter, were obtained with the *luxI* mutant only when autoinducer was included in the growth medium along with IPTG (Table 2). For the *luxI*$^+$ strain, the presence of IPTG alone was sufficient for generation of an induced level of luciferase (Table 2).

These results establish that in *E. coli*, autoinducer is required for *luxR* negative autoregulation at the transcriptional level. Repression of *luxR* promoter activity and induction of *lux* operon promoter activity thus both require autoinducer and LuxR. Since these activities appear to occur simultaneously (Tables 1 and 2), the proposed autoinducer-LuxR transcriptional activator complex (8, 9, 16) may require only a single DNA-binding site (2, 11). As discussed previously (5), results indicating negative autoregulation of *luxR* at the transcriptional level (Table 1) appear to be at variance with those of Engebrecht and Silverman (10). Using a *luxR* complementing plasmid with *luxR* under control of its native promoter, these investigators did not observe repression of β-galactosidase with *luxR*::Mu dI (transcriptional) fusions (10). However, repression of β-galactosidase was observed with *luxR*::Mu dII (translational) fusions located distal to the *luxR* promoter, and that repression required autoinducer (10). In the present study, as well as in a previous study (5), it is possible that removal of *luxR* from control by its native promoter and the use of a *cya* mutant helped reveal *luxR* negative autoregulation at the transcriptional level and its dependence on autoinducer (Table 1).

TABLE 2. Luciferase activity in *E. coli* PD200(pPD749) containing pJR551 or pHK555

Growth medium addition(s)	Luciferase activity units (10^3)	
	pJR551 (*luxI* mutant)	pHK555 (*luxI*$^+$)
None	13	10
cAMP (5 mM)	5	6
cAMP and autoinducer (0.2 μM)	160	170
cAMP and IPTG (1 mM)	150	4,340
cAMP, autoinducer, and IPTG	6,530	6,400

The data presented here support and extend a model for *lux* gene regulation in which transcriptional negative autoregulation of *luxR* was proposed (5), and they complement results of a study in which an autoinducer-dependent translational *luxR* negative autoregulation was demonstrated (10). Thus, expression of *luxR* in *E. coli* is negatively autoregulated at the transcriptional and at the translational levels. Furthermore, *luxR* expression is negatively controlled by the *luxI* gene product (9, 16) and is positively controlled at the transcriptional level by cAMP and CRP (4, 5). The various controls over *luxR* expression may be important at different times during growth of cells in culture and in natural environments, modulating the cellular concentration of LuxR and thereby functioning to turn on and turn off *lux* operon transcription at physiologically appropriate times. To examine the timing of these controls and their relative importance for *lux* operon expression, in vivo studies with *V. fischeri*, such as those recently conducted with *V. fischeri* regulatory mutants (3), would be valuable. In addition, further in vitro studies on the binding of the autoinducer-LuxR and cAMP-CRP complexes to *lux* regulatory region DNA (e.g., references 2, 11, and 16) should be helpful in examining a proposed transcriptional antagonism between these two regulatory complexes (5).

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Control of *Vibrio fischeri lux* Gene Transcription by a Cyclic AMP Receptor Protein-LuxR Protein Regulatory Circuit

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Expression of the *Vibrio fischeri* luminescence genes (*lux* genes) requires two transcriptional activators: the *V. fischeri luxR* gene product with autoinducer and the cyclic AMP (cAMP) receptor protein (CRP) with cAMP. It has been established that autoinducer and the *luxR* gene product are required for transcriptional activation of the *luxICDABE* operon, which contains a gene required for autoinducer synthesis and genes required for light emission. However, the role of cAMP-CRP in the induction of luminescence is not clear. We examined transcriptional control of the *lux* genes in *Escherichia coli*, using catabolite repression mutants carrying *lux* DNA-containing plasmids. Transcriptional fusions between the *lacZ* gene on Mu dI and *luxR* were used to assess *luxR* promoter activity, and the *luxAB* genes (which encode the two luciferase subunits) were used as a natural reporter of *luxICDABE* promoter activity. A plasmid containing *luxR* under control of the cAMP-CRP-independent *tac* promoter was constructed to direct the synthesis of the *luxR* gene product in cells containing compatible *luxR*::Mu dI insertion mutant plasmids. In *E. coli*, cAMP-CRP activated transcription of *luxR* and concurrently decreased *luxICDABE* transcription. In the presence of relatively high levels of the *luxR* gene product, cAMP and CRP were not required for induction of the *luxICDABE* operon. The *luxR* gene product in the presence of autoinducer activated transcription of the *luxICDABE* operon, as has been shown previously, and we demonstrate that it also decreased *luxR* transcription. Apparently, control of the *V. fischeri* luminescence genes involves a regulatory circuit in which cAMP and CRP activate *luxR* transcription and in turn the *luxR* gene product activates transcription of the operon responsible for light emission (*luxICDABE*). Furthermore, in *lux* gene regulation cAMP-CRP and autoinducer-LuxR protein appear to function as transcriptional antagonists.

Luminescence of *Vibrio fischeri*, the light organ symbiont of pinecone fish, requires autoinduction. The autoinducer [*N*-(3-oxo-hexanoyl)homoserine lactone] is a specific *V. fischeri* metabolite that activates transcription of the luminescence genes (*lux* genes) when it reaches a critical concentration of a few molecules per cell. Cells are permeable to autoinducer, so this molecule accumulates within *V. fischeri* and in the external medium at equal concentrations (7, 15, 18, 22). At high cell densities, autoinducer can accumulate and cause induction of the luminescence enzymes. This is thought to be the case in the light organ symbiosis in which *V. fischeri* occurs at densities of 10^9 to 10^{10} cells per ml of organ fluid. In environments in which *V. fischeri* density is low, such as seawater ($<10^3$ cells per ml), autoinducer does not accumulate and *V. fischeri* luminescence would not be expected (19, 21–23).

A 9-kilobase (kb) fragment of *V. fischeri* DNA that encodes all the functions for luminescence and contains regulatory elements sufficient for their expression in *Escherichia coli* has been isolated (8). The cloned DNA contains seven *lux* genes that constitute the *lux* regulon, organized as two divergently transcribed units (Fig. 1). One transcriptional unit contains *luxR*, which encodes a protein required for cells to respond to autoinducer (the LuxR protein). The other transcriptional unit contains *luxA* and *luxB*, which encode the α and β subunits of luciferase; *luxC*, *luxD*, and *luxE*, which encode proteins involved in synthesis of the aldehyde substrate for luciferase; and *luxI*, which is the only *V. fischeri* gene required for synthesis of autoinducer in *E. coli* (2, 8, 9). As a consequence of the arrangement of *lux* genes, autoinducer controls expression of *luxI*, creating a positive autoregulation of autoinducer synthesis (8). Furthermore, a recent publication indicates that expression of *luxR* is negatively autoregulated at a posttranscriptional level (10).

Besides control by autoinduction, *V. fischeri* luminescence is thought to be regulated by the cyclic AMP (cAMP) receptor protein (CRP) and cAMP (6, 12). Since cAMP and CRP mutants of *V. fischeri* have not been isolated, much of the existing information on cAMP-CRP control of *lux* transcription has been derived from studies with *E. coli* mutants containing the cloned *lux* genes (6). It is not clear from the existing evidence how cAMP and CRP activate luminescence in *E. coli*. Using a plasmid containing a Mu dI(*lacZ*) fusion in *luxR*, we previously demonstrated that in the absence of a functional *luxR* gene, cAMP and CRP stimulate transcription from the *luxR* promoter (6). Thus, it seems possible that cAMP and CRP activate *luxICDABE* indirectly by effecting an increase in the *luxR* gene product to a level above the threshold required for autoinduction. In this article, we present evidence in support of this hypothesis. A strategy was used that involved subcloning of *luxR* on a *tac* promoter vector and use of this construct to direct synthesis of relatively high levels of the *luxR* gene product in *E. coli* cAMP and CRP mutants containing the *luxICDABE* operon and *luxR*::Mu dI(*lacZ*). This also allowed us to examine the affect of the *luxR* gene product on transcription from the *luxR* promoter. Transcription of the *luxICDABE* operon in vivo was monitored by measuring luciferase content, and

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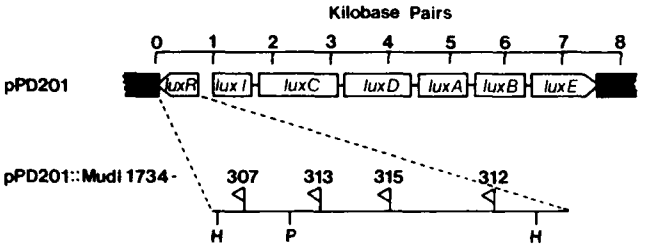

FIG. 1. Organization of *V. fischeri lux* genes and map locations of the *luxR*::Mu dI1734(*lacZ*) insertions used to study *luxR* promoter activity. (Top) Physical map of *lux* DNA in pPD201 showing the two divergently transcribed operons; the *luxR* operon and the *luxICDABE* operon. (Bottom) Restriction map of *luxR* in pPD201. H, *Hin*dIII; P, *Pst*I. The flags indicate the positions of four Mu dI1734 insertions and point in the direction of *lacZ* transcription.

transcription from the *luxR* promoter was monitored by measuring β-galactosidase content (6).

MATERIALS AND METHODS

Bacterial strains and culture conditions. All the strains used in this study are derivatives of *E. coli* K-12 and are listed in Table 1. For selection and screening of strains containing various plasmids, cells were transformed by the procedure of Hanahan (13) and plated on LB agar (24) supplemented as indicated and incubated for 1 to 2 days at 30°C. To monitor in vivo *luxR* and *luxICDABE* promoter activity, cultures were grown in LB broth or LB agar to which glucose (final concentration, 10 mM), Tris buffer (final concentration, 50 mM) (pH 7.5), and the appropriate antibiotics for plasmid maintenance (chloramphenicol [30 μg/ml] or chloramphenicol and ampicillin [80 μg/ml]) were added after sterilization. Cultures were grown in 3-ml volumes as described previously (6).

Construction of *luxR* insertion mutant plasmids. To construct plasmids containing the *lux* regulon with Mu dI1734 insertions in *luxR*, a 9-kb *Sal*I fragment of DNA containing the *lux* regulon was first removed from pJE202 and inserted into the *Sal*I site of pACYC184. Recombinant plasmids were selected by transformation of *E. coli* PD100 with the ligation mixture followed by plating of the transformed cells on LB agar containing chloramphenicol. An appropriate plasmid, pPD201, was obtained by screening transformants for luminescence (indicating the presence of the *lux* regulon) and ampicillin sensitivity (indicating the absence of pJE202). Mu dI1734 insertions in the *luxR* gene of pPD201 were then generated by the method of Castilho et al. (3). *E. coli* PD1734 was transformed with pPD201, Mu transposition was heat induced, and the resulting lysate was used to transduce *E. coli* PD100, with selection on LB agar containing chloramphenicol and kanamycin (20 μg/ml). Transductants which were not luminescent were isolated and screened for high-level expression of β-galactosidase on LB agar containing 5-bromo-4-chloro-3-indoyl-β-galactoside (X-gal) (suggestive of alignment of the *lacZ* gene of Mu dI with an active promoter). To identify *luxR*::Mu dI1734 insertions specifically, we used plasmid DNA from each of 85 strains containing pPD201 *lux*::Mu dI insertions to transform *E. coli* DH1 containing a *tac* promoter-*luxR* complementing plasmid, pPD749 (see below for construction), with selection on LB agar plus chloramphenicol and ampicillin followed by screening for luminescence in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM). Of the 85 plasmids examined by this method, 16 appeared to contain Mu dI insertions in *luxR*. To determine the orientation of the insertions and their positions in *luxR*, each of the 16 *luxR*::Mu dI insertion plasmids was digested with *Sal*I, *Eco*RI and *Bgl*II, and *Pst*I. Restriction maps of Mu dI1734 (provided by N. Kent) and the *lux* regulon (8) were used to estimate fragment sizes. Examination of the digestion fragments by agarose gel electrophoresis allowed mapping of the insertions with an accuracy of ±80 base pairs. Of the 16 *luxR*::Mu dI insertions examined, 8 were found to have *lacZ* in alignment with the *luxR* promoter, and 4 that had insertions

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli K-12		
JM109	F′ *lacI*^{q}	M. Weiner (25)
DH1	*recA1 hsdR17*	CGSC[b] (13)
PD1734	POI1734 [Mu cts, Mu dI1734 (Km^r *lacZYA*)] *recA56*	3. P. Dunlap, unpublished data
PD100	*cya*$^+$ *crp*$^+$ Δ(*argF-lac*)*U169*	6
PD200	Δ*cyaA-2 crp*$^+$ Δ(*argF-lac*)*U169*	6
PD300	*cya*$^+$ Δ*crp-3* Δ(*argF-lac*)*U169*	6
Plasmid		
pJE202	pBR322 with 9-kb *Sal*I fragment of *V. fischeri* DNA (*luxR luxICDABE*) Ap^r	J. Engebrecht (8)
pACYC184	Cm^r Tc^r	M. Weiner (4)
pHK555	pACYC184 with *luxICDABE luxR*::Mu Δ(*c, nerAB*) dI (Km^r *lacZYA*) Cm^r	16
pPD201	pACYC184 with *Sal*I *lux* fragment from pJE202, Cm^r	This study
pPD307	pPD201, *luxR*::Mu dI1734	This study
pPD312	pPD201, *luxR*::Mu dI1734	This study
pPD313	pPD201, *luxR*::Mu dI1734	This study
pPD315	pPD201, *luxR*::Mu dI1734	This study
pHK705	pUC18 with 1-kb *luxR* fragment, Ap^r	16
pKK223-3	*tac* promoter vector, Ap^r	1
pPD723	pKK223-3 with *luxR* fragment from pHK705, Ap^r	This study
p*lacI*^{q}	pACYC184 with 1.2-kb *lacI*^{q} fragment, Tc^r	L. Zumstein (1)
pPD749	pPD723 with *lacI*^{q} fragment from p*lacI*^{q}, Ap^r	This study

[a] Ap^r, Ampicillin resistant; Cm^r, chloramphenicol resistant; Tc^r, tetracycline resistant.
[b] CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

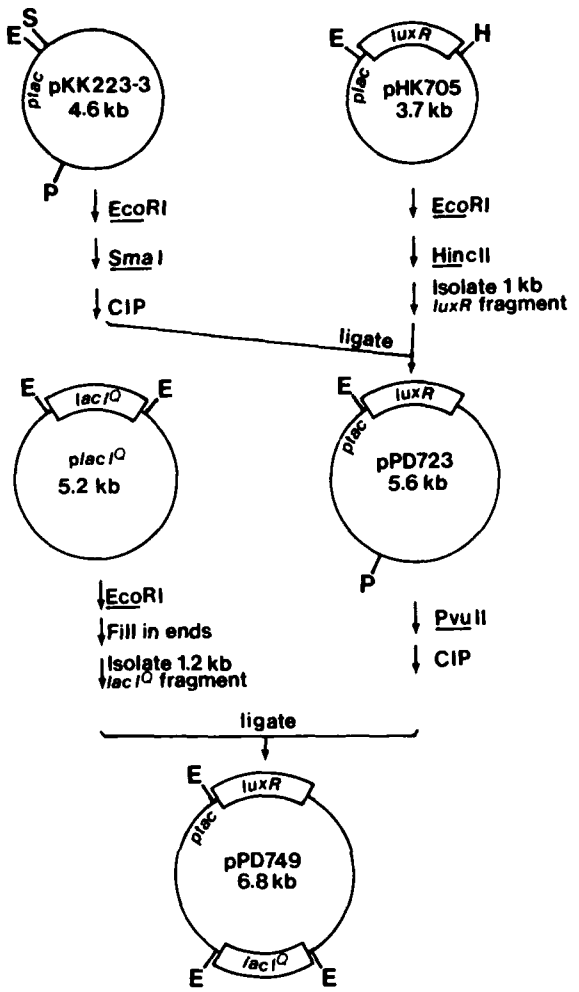

FIG. 2. Construction of the *luxR* complementing plasmid, pPD749. Restriction enzyme cleavage sites: E, *Eco*RI; H, *Hin*dIII; S, *Sma*I; and P, *Pvu*II. P_{lac} and P_{tac} indicate the *lac* promoter and *tac* promoter, respectively. CIP indicates treatment with calf intestinal phosphatase. The 1-kb *luxR* fragment and the 1.2-kb $lacI^q$ fragment were isolated after separation by electrophoresis through low-melting-point agarose.

widely separated from each other were chosen for further studies (Fig. 1).

Construction of plasmid containing *tac* promoter-controlled *luxR* gene. The construction of pPD749, the *luxR* complementing plasmid used in this study, is depicted in Fig. 2. The *luxR* gene was excised from pHK705, removing the *lac* ribosome-binding site and translation initiation sequence, and this gene was cloned downstream of the pKK223-3 *tac* promoter. An appropriate recombinant plasmid was obtained by transformation of *E. coli* JM109 containing pHK555 (*luxICDABE' luxR*), using ampicillin resistance (Ap^r) as a selectable marker and screening for transformants which were luminescent when grown in the presence of IPTG (1 mM), indicating complementation of the pHK555 *luxR* mutation by a *tac* promoter-controlled *luxR* gene. A plasmid from one of the transformants, designated pPD723, was chosen for further studies. A DNA sequence analysis (C. Countryman and T. Baldwin, personal communication) indicated that pPD723 contained only 15 base pairs of *lux* DNA 5′ to the *luxR* translational start site (5, 11). This plasmid did not contain the native *luxR* transcriptional start nor did it contain the *lux* DNA matching the consensus sequence for cAMP-CRP recognition (5, 11).

To permit IPTG-regulated expression of the *tac-luxR* construct in *E. coli* PD100, PD200, and PD300, which do not contain a *lacI* gene, we subcloned the $lacI^q$ gene into pPD723 to form pPD749 (Fig. 2). To obtain pPD749, we transformed *E. coli* PD410 (*lacI*; P. V. Dunlap, unpublished data) containing pHK555 with the pPD723-$lacI^q$ ligation mixture and plated it on LB agar plus chloramphenicol and ampicillin. Colonies that did not appear luminous upon visual examination were transferred to IPTG agar plates to identify transformants that were luminous upon induction of the *tac* promoter. One such transformant harbored pPD749, which was purified and used in our subsequent investigations. Restriction mapping was used to confirm the plasmid constructions.

Determination of cellular luminescence, luciferase activity, and β-galactosidase activity. The light-measuring equipment and standard to calibrate the equipment have been described previously (6, 12, 14), as have the procedures for measuring luminescence of broth cultures (6). Permanent records of colony luminescence on agar plates were obtained by exposing X-ray film to the agar plates. A neutral-density filter (25% transmittance) was placed between the film and the plates, and exposure time was 20 s. The procedure for measuring luciferase activity in cell extracts involved a reaction with excess flavin mononucleotide, decanal, and oxygen (20) as described previously (6). β-Galactosidase activity was measured by the $CHCl_3$-sodium dodecyl sulfate method described by Miller (17). As described previously, cells for β-galactosidase activity measurements and for luciferase activity measurements were harvested from cultures at an optical density at 660 nm of 1.0 (6).

Chemicals. Antibiotics, cAMP, decanal, flavin mononucleotide, IPTG, Tris, and X-gal were purchased from Sigma Chemical Co. (St. Louis, Mo.). *N*-(3-Oxo-hexanoyl)homoserine lactone (autoinducer) was synthesized and supplied by H. B. Kaplan and A. Eberhard.

RESULTS

CRP and cAMP are not required for induction of luciferase synthesis in the presence of sufficient *luxR* gene product. *E. coli* Δ*cyaA* mutants carrying pJE202 (*luxICDABE luxR*) required the addition of cAMP to the growth medium for luminescence, and Δ*crp* mutants were not brightly luminous even in the presence of added cAMP (6) (Fig. 3). This demonstrated that cAMP and CRP are required for activation of the *luxICDABE* operon on pJE202. This cAMP-CRP requirement may be indirect in that these effectors may act by inducing the synthesis of the *luxR* gene product which in turn is needed for *luxICDABE* activation (6) (see Introduction). When the *luxR* gene was supplied under control of the cAMP-independent *tac* promoter (on pPD749) to *E. coli* Δ*cyaA* or Δ*crp* mutants containing the *luxICDABE* operon on pHK555 [*luxICDABE luxR*::Mu dI1681(*lacZ*)], luminescence was not dependent on cAMP or CRP; luminescence was dependent only on IPTG, the inducer of the *tac* promoter (Fig. 3).

To quantitate the effects of cAMP and CRP on transcription of the *luxICDABE* operon in the presence of *luxR*, we measured luciferase activities in extracts of *E. coli* strains carrying pHK555 and pPD749 (Table 2). In the presence of IPTG, induced levels of luciferase were achieved in the Δ*crp* mutant and in the Δ*cyaA* mutant with or without added

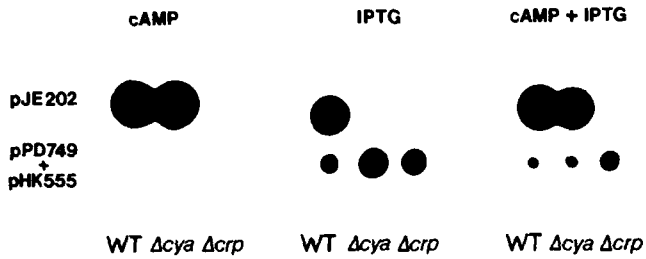

FIG. 3. Relationship between colony luminescence, cAMP, CRP, and *luxR* protein. Three agar plates, one containing cAMP (5 mM), one containing IPTG (1 mM), and one containing cAMP and IPTG, were spotted with small drops of six different bacterial cultures. Each plate was spotted with *E. coli* PD100 (*cya*$^+$ *crp*$^+$), PD200 (Δ*cyaA*), and PD300 (Δ*crp*) containing either pJE202 or pPD749 and pHK555. After 24 h at 30°C, colony luminescence was recorded on X-ray film as described in Materials and Methods. WT, Wild type.

cAMP. In the absence of IPTG, LuxR protein concentrations were apparently not sufficient for activation of luciferase synthesis even in the *cya*$^+$ *crp*$^+$ strain. Since pHK555 contains a functional *luxI* gene, the *E. coli* strains in these experiments were capable of synthesizing autoinducer, and exogenously added autoinducer had little effect on the synthesis of luciferase (Table 2). It should be noted here that in the presence of IPTG these strains exhibited the characteristic autoinducer response; luciferase activity remained at a constant uninduced level for the first 4 to 5 h of growth and was then induced to a level about 100-fold greater than the uninduced level. The induction lag could be overcome by the addition of autoinducer (0.2 μM) to the growth medium (data not shown). These in vivo experiments demonstrate that in the presence of sufficient *luxR* gene product, *luxICDABE* transcription did not require cAMP and CRP.

Effects of cAMP and CRP on *lux* promoter activity. Based on experiments in which β-galactosidase levels in *E. coli* Δ*cyaA* and Δ*crp* mutants containing pJE455 [*luxICDABE luxR*::Mu dI1681(*lacZ*)] were monitored, it was concluded that transcription from the *luxR* promoter is stimulated by cAMP-CRP (6). This, taken together with the results presented in Table 2 and Fig. 3, suggests that for the intact *lux* regulon, cAMP and CRP are required for the establishment of levels of the LuxR protein sufficient for induction of luminescence. This hypothesis was examined further by addressing two remaining questions. (i) Is the transcriptional stimulation from the *luxR* promoter evident regardless of the position of the *lacZ* insertion in *luxR* (only one transcriptional fusion was examined previously)? (ii) Does transcriptional activation of *luxR* by cAMP and CRP occur in the presence of a functional LuxR protein (cells in the previous study were *luxR*)?

TABLE 2. Luciferase activity in *E. coli* catabolite repression mutants containing pHK555 and pPD749

Addition(s)	Luciferase activity units (10^5)		
	PD100 (*cya*$^+$ *crp*$^+$)	PD200 (Δ*cyaA crp*$^+$)	PD300 (*cya*$^+$ Δ*crp*)
None	10	45	15
cAMP (5 mM)	10	25	15
IPTG (1 mM)	2,800	5,600	3,300
cAMP and IPTG	3,300	3,400	3,500
cAMP, IPTG, and autoinducer (0.2 μM)	3,600	3,800	5,600

TABLE 3. Influence of cAMP on β-galactosidase synthesis and luciferase synthesis in *E. coli* PD200 (Δ*cyaA*) containing *lux* plasmids with Mu dI(*lacZ*) insertions in *luxR*

Insertion plasmid[a]	β-Galactosidase activity units		Luciferase activity units (10^5)	
	−cAMP	5 mM cAMP	−cAMP	5 mM cAMP
pPD312	80	960	11.8	4.8
pPD315	30	320	5.7	3.6
pPD313	40	500	8.1	3.9
pHK555	30	360	7.2	4.0
pPD307	20	130	5.1	2.1

[a] For positions of Mu dI insertions in *luxR*, see Fig. 1 and text.

To examine the relationship between cAMP stimulation of *luxR-lacZ* transcription and the position of the *lacZ* fusion, we measured β-galactosidase activity in *E. coli* PD200 (Δ*cyaA*) with each of four *luxR*::Mu dI1734 insertion plasmids or with one *luxR*::Mu dI1681 insertion plasmid (Table 3). The map positions of each of the Mu dI1734 insertions are shown in Fig. 1. There appears to be some correlation between distance of the *lacZ* insertion from the *luxR* promoter and the level of transcription. More importantly, in every case cAMP stimulated transcription from the *luxR* promoter about 6- to 12-fold (Table 3). In these experiments, the presence of cAMP in the growth medium did not stimulate growth (data not shown). These data confirm our previous conclusion that cAMP stimulates transcription from the intact *luxR* promoter (6). Also, the cAMP stimulation is independent of the *lux* DNA orientation in the plasmid—compare the results with pPD313 to those with pHK555, the *luxR*::Mu dI1681 insertion plasmid; the insertions map at approximately the same position in *luxR* but the *lux* DNA in pHK555 is oriented in the vector DNA opposite to its orientation in pPD313.

In the experiment described above, transcription from the *luxICDABE* promoter was monitored concurrently with transcription from the *luxR* promoter by measuring luciferase activity (Table 3). Consistent with our previous study (6), cAMP decreased transcription from this promoter by about 50% in these *luxR* cells.

To examine the influence of cAMP and CRP on the *luxR* promoter in the presence of the LuxR protein, we used the *tac* promoter-controlled *luxR* on pPD749 to complement the *luxR*::Mu dI(*lacZ*) mutation on pHK555. As in the experiments described above, the level of β-galactosidase was measured as an indication of *luxR* promoter activity. Without induction of the *tac* promoter-controlled *luxR* gene by IPTG, cAMP stimulated transcription of *lacZ* from the *luxR* promoter in the *E. coli* Δ*cyaA* mutant and the *cya*$^+$ *crp*$^+$ strain containing pHK555 and pPD749. The basal level of *luxR* promoter activity was higher in the parent, presumably because of the ability of this strain to synthesize cAMP. The *luxR* promoter activity was not affected by the addition of cAMP to the *E. coli* Δ*crp* mutant (Table 4). This is in agreement with our conclusion that cAMP and CRP can stimulate transcription from the *luxR* promoter when the LuxR protein is not abundant (6). When IPTG was added to induce synthesis of the LuxR protein, the level of *luxR* promoter activity was affected (see below). Nevertheless, for the parent strain and particularly for the Δ*cyaA* mutant (PD200), it is evident that cAMP stimulated *luxR* promoter activity in the presence of relatively high levels of the LuxR protein, as it did in the absence of this protein. For the

TABLE 4. β-Galactosidase activity in *E. coli* catabolite repression mutants containing pHK555 and pPD749

Addition(s)	β-Galactosidase activity units		
	PD100 (*cya*$^+$ *crp*$^+$)	PD200 (Δ*cyaA crp*$^+$)	PD300 (*cya*$^+$ Δ*crp*)
None	170	35	45
cAMP (5 mM)	690	470	40
IPTG (1 mM)	95	15	25
cAMP and IPTG	160	150	25
cAMP, IPTG, and autoinducer (0.2 μM)	100	70	25

Δ*cyaA* mutant, the cAMP stimulation was 10-fold (Table 3), comparable to the magnitude of stimulation in the absence of LuxR protein (Table 3). In PD300, the Δ*crp* strain, addition of cAMP did not influence the level of transcription from the *luxR* promoter in IPTG-grown cells (Table 4). Apparently, transcriptional activation of the *luxR* promoter by cAMP did occur in the presence of a functional LuxR protein and this stimulation depended on CRP. Similar results for cAMP control of *luxR* promoter activity were obtained with PD200 (Δ*cyaA*) containing pPD749 plus each of the other four *luxR*::Mu dI(*lacZ*) insertion plasmids: pPD312, pPD315, pPD313, and pPD307 (data not shown).

Influence of *luxR* gene product on *luxR* promoter activity. The ability to control synthesis of the LuxR protein with the cAMP-CRP-independent *tac* promoter on pPD749 and to monitor transcription of the *luxR-lacZ* fusion on pHK555 in Δ*cyaA* and Δ*crp* backgrounds enabled us to study the influence of the LuxR protein on *luxR* promoter activity in vivo. We sought to examine this question because it has been reported that *luxR* is negatively autoregulated, apparently at a posttranscriptional level rather than at the level of transcription (10). The evidence presented here indicates the *luxR* gene product was involved in a negative autoregulation of transcription from the *luxR* promoter (Table 4). When cultures of the *cya*$^+$ *crp*$^+$ strain or either of the mutants were grown in the presence of IPTG to induce synthesis of the LuxR protein, the level of transcription from the *luxR* promoter was about 50% of that when cells were grown without IPTG. The *cya*$^+$ *crp*$^+$ strain and the Δ*cyaA* mutant grown with cAMP and IPTG exhibited only about 25% of the *luxR* promoter activity exhibited by cAMP-grown cells. When saturating concentrations of autoinducer (15) were added with IPTG and cAMP, levels of transcription from the *luxR* promoter were about 15% of the levels in cAMP-grown cells of either the parent or the Δ*cyaA* mutant. The effect of added autoinducer suggests that for maximum negative regulation of the *luxR* promoter by the LuxR protein, autoinducer is required. However, because the cells in these experiments possessed an intact *luxI* gene on pHK555 and thus could synthesize autoinducer, the role of autoinducer in *luxR* regulation awaits clarification. Similar results for *luxR* negative autoregulation were obtained with PD200 (Δ*cyaA*) containing pPD749 plus each of the other four *luxR*::Mu dI(*lacZ*) insertion plasmids: pPD312, pPD315, pPD313, and pPD307 (data not shown).

DISCUSSION

The results of this study extend our understanding of the basis for cAMP-CRP control of the *V. fischeri* luminescence regulon and provide evidence for negative autoregulation of *luxR* at the level of transcription. Although expression of the *luxICDABE* operon in *E. coli* containing the intact *lux* regulon depended on cAMP and CRP (6) (Fig. 3), induced levels of expression of this operon did not require cAMP or CRP when sufficient concentrations of the LuxR protein were produced in *E. coli* by a *tac* promoter-controlled *luxR* structural gene (Fig. 2; Table 2). Thus, in *E. coli*, induction of *luxICDABE* did not require cAMP-CRP directly. Taken together with the observation that the *luxR* promoter is activated by cAMP-CRP (6) (Tables 3 and 4), this indicates that a primary role of cAMP and CRP in *lux* gene regulation is to activate transcription of *luxR*, thereby increasing the concentration of the LuxR protein to a level sufficient for it to serve in conjunction with autoinducer as a *luxICDABE* transcriptional activator.

The results of experiments in which a *luxR-lacZ* transcriptional fusion was used to monitor *luxR* promoter activity in the presence of the *tac-luxR*-containing plasmid indicated that in *E. coli* the *luxR* gene product repressed transcription from the *luxR* promoter and that cAMP-CRP partially relieved that repression (Table 4). Regulation of the *luxR* promoter appears substantial; i.e., the fully repressed levels in IPTG-grown cells of the Δ*cyaA* mutant were only about 3% of the fully induced levels exhibited by cAMP-grown cells (Table 4). Our results appear to be at variance with those of Engebrecht and Silverman (10). Using a *luxR* complementing plasmid with *luxR* under control of its native promoter, these investigators did not observe repression of β-galactosidase in *luxR*::Mu dI (transcriptional) fusions. However, in *luxR*::Mu dII (translational) fusions located distal to the *luxR* promoter, a decrease in β-galactosidase activity in the presence of the *luxR* complementing plasmid was observed (10). It is possible that removal of *luxR* from control by its native promoter and the use of catabolite repression mutants helped reveal the negative autoregulation of *luxR* at the transcriptional level (Table 4). These findings do not preclude the possibility that there is also posttranscriptional negative autoregulation of *luxR* as has been suggested elsewhere (10).

Our investigations of *lux* gene control in *E. coli* lead to the proposal that CRP and the LuxR protein are transcriptional antagonists. Transcription from the *luxR* promoter was activated by cAMP-CRP, whereas transcription from the *luxICDABE* promoter was repressed (Tables 3 and 4). The LuxR protein, apparently together with autoinducer, repressed transcription from the *luxR* promoter (Table 4) and activated *luxICDABE* transcription (Table 2). The evidence for autoinducer involvement in the *luxR* negative autoregulation is not firm, but it has been well established that autoinducer is required for induction of the *luxICDABE* operon (6, 8, 9).

The data presented here led us to develop a working model for transcriptional control of the *lux* regulon (Fig. 4) that is consistent with all other published data of which we are aware. The model indicates that there is a region of approximately 220 base pairs between the *luxR* and *luxI* open reading frames (5, 11) and that together with cAMP, CRP binds to a site about midway between the two open reading frames. Placement of the CRP-binding site is based on identification of a DNA sequence which compares favorably with the consensus sequence for *E. coli* CRP-binding sites (5, 11). According to the model, CRP binding activates transcription of *luxR*, thus driving up the concentration of the LuxR protein, and decreases transcription of *luxICDABE* to an even lower level than in the absence of the LuxR protein. The small available quantities of the *luxI* gene product catalyze the synthesis of autoinducer at a slow rate.

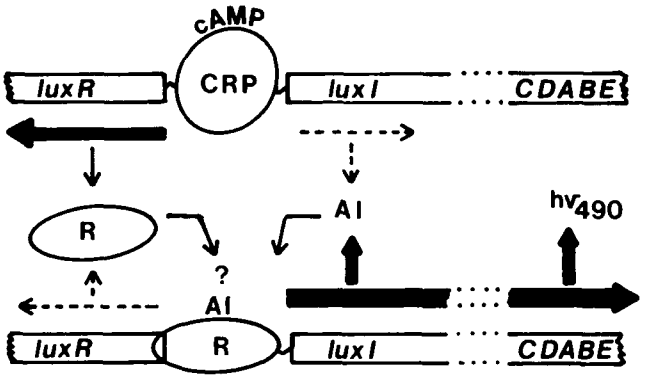

FIG. 4. Model for transcriptional regulation of the *V. fischeri lux* regulon. CRP and cAMP bind to the region between *luxR* and *luxI*, activate transcription of *luxR* (indicated by the heavy arrow) and decrease transcription of *luxICDABE* (indicated by the light arrow). The activation of *luxR* transcription increases the concentration of the LuxR protein (R) to a sufficient level for interaction with the low concentration of autoinducer (AI) that has accumulated owing to basal expression of the *luxI* gene and the resulting low activity of the *luxI* gene product. The LuxR protein and autoinducer then bind to a region upstream of the *luxICDABE* promoter, activating transcription of *luxICDABE*. This leads to increased levels of autoinducer and to induced levels of luminescence (hv_{490}). Since LuxR protein and autoinducer also deactivate transcription of *luxR*, the level of LuxR protein will be fine tuned continually. As this protein becomes limiting at any given autoinducer concentration, the apparent repression of *luxR* will be relieved and the cAMP-CRP complex will bind to the DNA, thus activating transcription of *luxR*.

Nevertheless, autoinducer will accumulate to concentrations of 5 to 10 nM, which are sufficient for an undefined interaction with the LuxR protein and induction of the *luxICDABE* operon (8, 15). We propose that the LuxR protein and autoinducer also deactivate *luxR* transcription (Table 4), thereby counterbalancing the effect of cAMP and CRP. Thus, the LuxR protein will be adjusted continually to levels appropriate for activity at any given concentration of autoinducer. This model is based in large part on experiments with *E. coli* in which *lux* genes were provided in a variety of dosages. Further studies with chromosomal genes in a *V. fischeri* background will serve to test the validity of the model.

DNA sequencing has revealed a 20-base-pair palindrome at −62 with respect to the translational start of *luxI* which is a potential site for binding of a regulatory protein and may represent the LuxR protein-binding site (5). It should be noted, however, that in *E. coli*, overproduction of the LuxR protein directed by a high-copy-number *tac-luxR*-containing plasmid is suppressed by the presence of the *luxICDABE* operon on a low-copy-number plasmid (16). This suggests that there is a *luxR* operator within the *luxR* structural gene or that an additional control element such as the posttranscriptional control postulated by Engebrecht and Silverman (10) is significant (5). The question mark above the autoinducer-LuxR protein-DNA complex in the model (Fig. 4) is meant to indicate several points. Autoinducer binding to the LuxR protein has not been demonstrated, and although it has been demonstrated that the LuxR protein can bind DNA, specific binding to *lux* DNA has not been demonstrated (16). Finally, there is some evidence to suggest that autoinducer and the LuxR protein do not act alone to suppress the synthesis of the LuxR protein (10, 16). The limited data are consistent with the idea that the *luxI* gene product has a dual function: it is required for autoinducer synthesis and it is involved more directly at some level (perhaps a posttranscriptional level) in *luxR* regulation (10, 16). Clearly, this idea remains to be tested.

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